FEBS Letters 586 (2012) 1300–1305

FEBS Letters

journal homepage: www.FEBSLetters.org

Ceramide kinase deficiency improves diet-induced obesity and insulin resistance

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ARTICLE INFO

Article history:
Received 16 January 2012
Revised 16 February 2012
Accepted 15 March 2012
Available online 27 March 2012

Edited by Laszlo Nagy

Keywords:
Ceramide kinase
Ceramide 1-phosphate
Sphingolipid
Obesity
MCP-1

ABSTRACT

Ceramide kinase (CERK) is an enzyme that phosphorylates ceramide to produce ceramide 1-phosphate. Recently, evidence has emerged that CERK has a role in inflammatory signaling of immune cells. Since obesity is accompanied by chronic, low-grade inflammation, we examined whether CERK might be involved using CERK-null mice. We determined that CERK deficiency suppresses diet-induced increases in body weight, and improves glucose intolerance. Furthermore, we demonstrated that CERK deficiency attenuates MCP-1/CCR2 signaling in macrophages infiltrating the adipose tissue, resulting in the suppression of inflammation in adipocytes, which might otherwise lead to obesity and diabetes.

1. Introduction

The enzyme ceramide kinase (CERK) catalyzes the conversion of ceramide to ceramide 1-phosphate (C1P). In 2002, the CERK gene was cloned [1]. This breakthrough stimulated many groups to study CERK and C1P, leading to discoveries in mast cell activation, eicosanoid signaling, and anti-apoptotic signaling [2]. Studies also determined that CERK is involved in cellular inflammatory signaling [3].

Obesity, which has grown to epidemic proportions throughout the world, is an important risk factor for the development of diabetes and cardiovascular disease [4]. Chronic and low-grade inflammation is associated with the initial steps of obesity; occurring in the absence of any infection of microorganisms it is termed "sterile inflammation" [5]. In this study, we investigated CERK involvement in sterile inflammation and obesity, which is associated with diabetes.

2. Materials and methods

2.1. Animal studies

Generation of CERK-/- mice was previously described [6]. For diet-induced obesity (DIO), the CERK-/- mice and wild type mice (C57BL/6J) were fed a high fat diet (60% kcal from fat; 58Y1, Test-Diet, Richmond, IN) from 4 to 15 weeks of age, and body weights were measured weekly. Glucose tolerance tests (GTT) were performed as described previously [7].

2.2. Immunohistochemical studies

For histological studies, mice epididymal adipose tissues were fixed with 4% paraformaldehyde, and 8-μm-thick frozen sections were prepared as described previously [6]. The sections were stained with hematoxylin and eosin. For immunohistochemistry, fats were first removed from the sections using a combination of ethanol and xylene. Endogenous peroxidase was quenched with 3% H_2O_2, and anti F4/80 antibody (eBioscience) and anti-rat IgG-horse radish peroxidase (GE Healthcare) were used to stain macrophages by Vectastain Elite ABC peroxidase kit (Vector laboratories) according to manufacture's instructions. Images were obtained using a Axioskop 2 plus microscope (Carl Zeiss), and adipocyte cell

Abbreviations: Cer, ceramide; CERK, ceramide kinase; C1P, ceramide 1-phosphate; MCP-1, monocyte chemoattractant protein-1; CCR2, C-C motif chemokine receptor-2; FBS, fetal bovine serum; BMDM, bone-marrow derived macrophage

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http://dx.doi.org/10.1016/j.febslet.2012.03.032

areas were measured using the manufacture's software, AxioVision 4 (Carl Zeiss).

2.3. Real-time PCR

Total RNA was extracted from mice epididymal adipose tissues using a combination of TRIsol® and PureLink™ RNA Mini kits (Invitrogen), according to instructions. After first strand cDNA synthesis, real-time PCR was performed as described previously [7]. The transcript levels were normalized with hypoxanthine guanine phosphoribosyl transferase (HPRT). Primer sets used in this study were as follows: perilipin, sense 5′-GATGAGAGCCATGACGACCAGA and antisense 5′-TGTGTACCACACCACCCAGGA; F4/80, sense 5′-TCTGTTCACCATCATCAATGTCC and antisense 5′-GACACTTCAGTGCTTTCACTTTCAA; IL6, sense 5′-CCACTTCACAAGTCGGAGGCTTA and antisense 5′-GCAAGTGCATCATCGTTGTTCATAC; TNFα, sense 5′- AAGCCTGTAGCCCACGTCGTA and antisense 5′-GGCACCACTAGTTGGTTGTCTTTG; MCP1, sense 5′- GCATCCACGTGTTGGCTCA and antisense 5′-CTCCAGCCTACTCATTGGGATCA; Hprt1, sense 5′-TTGTTGTTGGATATGCCCTTGACTA and antisense 5′-AGGCAGATGGCCACAGGACTA; adiponectin, sense 5′-GTCAGTGGATCTGACGACACCAA and antisense 5′-ATGCCTGCCATCCAACCTG; GLUT4, sense 5′-CTGTAACTTCATTGTCGGCATGG and antisense 5′-AGGCAGCTGAGATCTGGTCAAAC; IR, sense 5′-CAGCTCGAAACTGCATGGTTG and antisense 5′-GGTGACATCCACCTCACAGGAA.

2.4. Preparation of bone marrow-derived macrophages (BMDM)

Samples of bone marrow from the femurs of 6-week old CERK-/- and WT male mice were placed in RPMI 1640 medium (Sigma) containing 10% fetal bovine serum (FBS), penicillin/streptomycin, and recombinant mouse macrophage colony stimulating factor (M-CSF, 100 ng/ml, Wako Pure Chemicals) as described elsewhere [8]. Cells were maintained in culture at 1×10^6 cells/ml for 5 days. Prior to experiments, BMDM purity was monitored by flow cytometry using an FITC-labeled antibody for the macrophage-specific marker F4/80 (BD Bioscience, CA).

2.5. Phagocytosis, adhesion, and cell-migration assay of BMDM

For phagocytosis assays, Texas Red-conjugated zymosan A bioparticles (Invitrogen) were opsonized with human serum as described elsewhere [9]. BMDM were seeded on 60 mm dishes and cultured for 5 days. After a 15 min incubation of the cells with the opsonized zymosan particles at 37 °C, particles incorporated into the cells were counted using a confocal laser scanning microscope (LSM510, Carl Zeiss). For cell adhesion assays, 96 well plates were coated overnight at 4 °C with 10 μg/ml fibronectin (Sigma) or 5 μg/ml laminin (Sigma). Wells were washed with phosphate buffered saline (PBS), and 1×10^5 BMDM were seeded into each well. After 5 days, the amount of cellular adhesion was monitored using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). Cell migration assays were performed using Transwell Boyden Chambers (pore size 8.0 μm, Costar). The lower side of each chamber was precoated with fibronectin (10 μg/ml) overnight at 4 °C, washed with PBS, and air-dried. Serum-starved cells (1×10^5 cells) were added to chamber, and the chambers were placed in 24-well dishes containing medium with 50 ng/ml MCP-1 or 10% fetal bovine serum (FBS) and incubated for 3-h at 37 °C. Migrated cells were visualized by staining with Crystal Violet (0.1% in 0.1 M borate (pH 9.0), and 2% ethanol) then counted.

2.6. SDS–PAGE and Western blotting

SDS–PAGE and Western blotting were performed according to standard methods described previously [7], using anti-CCR2 mouse IgG (Santa Cruz Biotechnology), anti-ERK (p42/44) MAP kinase rabbit IgG (Cell Signaling Technology), or anti-phosphorylated ERK (pERK) mouse IgG (Santa Cruz Biotechnology) as the primary antibody, and an anti-mouse IgG-HRP antibody (GE Healthcare), or anti-rabbit IgG-HRP antibody (GE Healthcare) as the secondary antibody. Bands were detected using a combination of an ECL plus kit (GE Healthcare) and X-ray film exposure.

3. Results

3.1. CERK deficiency suppresses diet-induced increases in body weight, and improves glucose intolerance

We previously reported that ceramide kinase (CERK) might act as a modulator for chronic activation of mast cells [10], and others have indicated that ceramide 1-phosphate (C1P), is a mediator of eicosanoid synthesis [11]. Thus, there is emerging evidence that CERK is involved in cellular inflammatory processes [2]. A recent study suggested that obesity is accompanied by a state of chronic, low-grade inflammation that contributes to insulin resistance and type 2 diabetes [5]. We investigated a possible relationship between obesity and CERK using the diet-induced obesity (DIO) animal model. CERK-/- and wild type (WT) mice were fed a high-fat diet from 4 to 15 weeks of age. In this obesity model, 14–20 weeks of diet are sufficient to cause obesity and insulin-resistance [7]. As expected, the body weights of the WT mice were significantly increased (Fig. 1A). Interestingly, though, CERK deficiency strongly prevented the increase in body weight. We also examined the effect of CERK deficiency on glucose intolerance or insulin resistance (Fig. 1B). Glucose tolerance tests (GTT) reflected impaired blood glucose clearance in WT mice, as evidenced by a decreased ability to lower their blood glucose. However, the CERK-/- mice showed significantly lower glucose levels than in the WT animals, indicating that CERK deficiency increases insulin sensitivity.

3.2. CERK deficiency improves adipose function

Smaller adipocytes are usually observed with increased insulin sensitivity [12]. To examine the adipocytes of the test animals, we prepared sections of epididymal adipose and stained them with hematoxylin and eosin (Fig. 1C). We also measured the surface area of each adipocyte (Fig. 1D), and plotted its distribution (Fig. 1E). The results clearly indicate that the CERK-/- mice had a greater number of small adipocytes than did the WT mice.

To gain insight into the mechanisms behind the effects of CERK deficiency on diet-induced obesity and insulin resistance, we examined the expression levels of genes for proteins associated with obesity, using real-time quantitative PCR. For example, perilipin, a coat protein of lipid droplets in adipocytes, is abundant in small adipocytes [13]. The adipose tissue of CERK-/- mice expressed perilipin at levels higher than those observed in WT mice (Fig. 2A). Recent investigations suggest that obesity is associated with a state of chronic, low-grade inflammation in adipose tissue, which is known to produce inflammatory cytokines and chemokines [5]. Adipose tissue from WT mice expressed high levels of the inflammatory cytokines, IL-6 and TNFα, which have been implicated as playing a role in obesity. However, CERK deficiency strongly suppressed the elevation of these inflammatory cytokines (Fig. 2B, C). Additionally, we examined the expression levels of insulin receptor (IR), GLUT4 (major glucose transporter in adipose tissue), and adiponectin to discuss insulin-signaling of adipose tissue in these mice (Fig. 2F–H), and showed that CERK-deficiency improved HFD-induced decrease of IR, GLUT4, and adiponectin. These results indicated that the adipocytes of CERK-/- mice maintained normal insulin-signaling even after HFD-feeding.

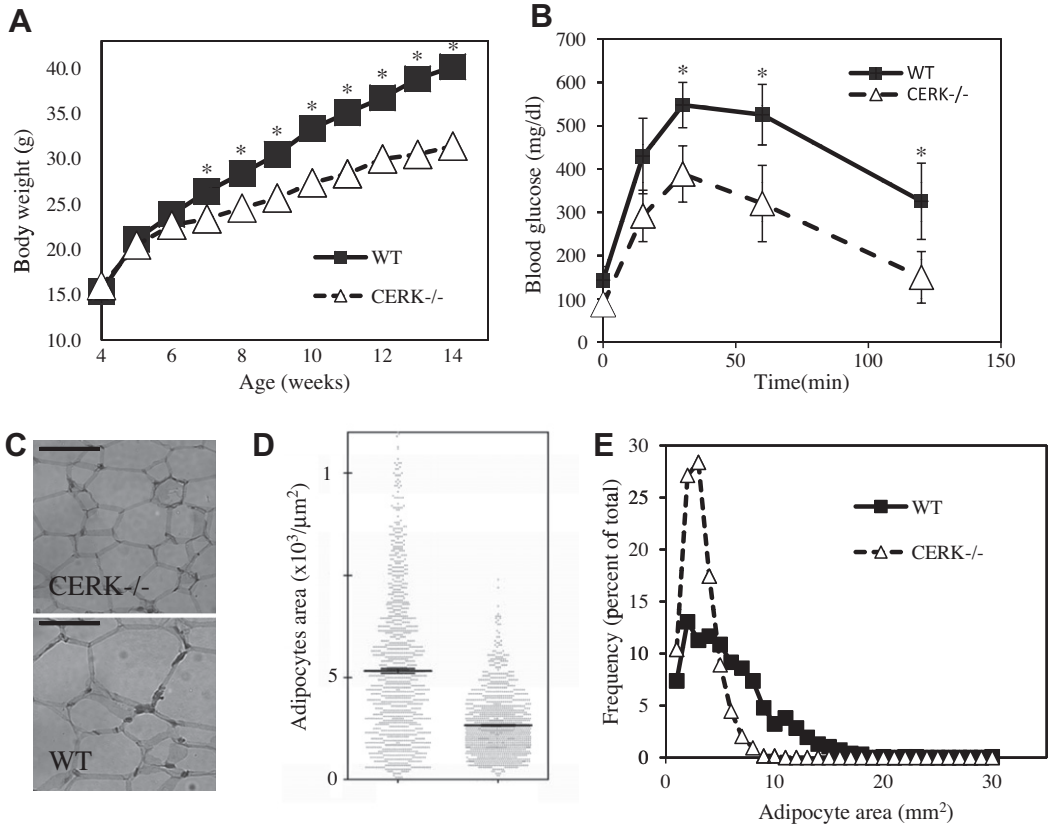

Fig. 1. Effects of CERK deficiency on diet-induced body weight gain and adipose tissue. Wild type (WT) and CERK-/- mice were fed a high-fat diet as described in Section 2. (A) Body weights increased significantly in the wild type (WT) mice but not in the CERK-/- mice. (B) Glucose tolerance tests (GTT) were performed on the mice in (A). (A–B) $n = 13$ and 10 for WT and CERK-/-, respectively. *, $P < 0.001$. (C) Hematoxylin and eosin staining indicates a greater number of small adipocytes in CERK-/- adipose tissues than in WT tissues. Image scale is 100 μm. (D and E) Cell surface areas of adipose tissues from WT and CERK-/- mice were measured, and the means (D) and distributions (E) were plotted. The data include 1235 cells from WT (five mice) and 1279 cells from CERK-/- mice (five mice). #, $P < 0.000001$.

3.3. CERK deficiency prevents infiltration of macrophages into adipose tissue

The inflammatory processes associated with obesity have not been fully established, though there is much interest in the role of adipose tissue macrophages in the inflammation characteristically observed in obesity [14]. Monocyte chemoattractant protein-1 (MCP-1) is known to induce macrophage infiltration into adipose tissue. Adipose tissue from CERK-/- mice expressed lower levels of MCP-1 than did tissue from WT mice (Fig. 2D). Additionally, the expression levels of a macrophage-specific gene, F4/80, in CERK-/- mice were nearly half those in WT mice, indicating that MCP-1-induced infiltration of macrophages into adipose is strongly inhibited by CERK deficiency.

A recent study revealed that macrophages recruited to adipose are localized around dead adipocytes, forming "crown like structures (CLS)" that produce additional chemokines (including MCP-1) and cytokines, resulting in worsening of inflammation and subsequent insulin resistance [15]. In the adipose of the WT mice, there were many adipocytes surrounded by macrophages forming CLS (Fig. 3A; arrow). The CLS-surrounded adipocytes reached nearly 20 percent of the total adipocytes counted (Fig. 3B). Surprisingly, there were almost no CLS in the adipose of CERK-/- mice (Fig. 4A, B).

3.4. CERK regulates MCP-1/CCR2 signaling in macrophages

We previously described that CERK is involved in the differentiation of leucocytes [16], and that it acts as a Ca^{2+} sensor of degranulation in mast cells [17]. With this in mind, we next examined whether CERK is involved in macrophage function using CERK-/- mouse bone marrow-derived macrophages (BMDM). BMDM were isolated and cultured as described in Section 2. After 5 days in culture, all progenitor cells had completely differentiated to F4/80-positive macrophages, indicating that macrophage differentiation is not impaired in the BMDM from CERK-/- (Fig. 4A). We next compared the phagocytosis (Fig. 4B), adhesion to extracellular matrix (Fig. 4C), and cell migration (Fig. 4D) of BMDM from WT and CERK-/- mice. Phagocytosis of zymosan particles (Fig. 4A) and adhesion of the cells to fibronectin and laminin did not differ between the BMDM from WT and CERK-/- mice.

MCP-1-induced cell migration was significantly reduced in BMDM from CERK-/- mice, but no such reduction was observed in serum-induced cell migration (Fig. 4D). Expression of CCR2, a receptor for MCP-1, was similar in BMDM from both mice (Fig. 4E), yet MCP-1-induced phosphorylation of ERK was suppressed in the BMDM from CERK-/- mice. Addition of short-chain C8-C1P has been demonstrated to induce phosphorylation of ERK in macrophages [18], therefore CERK/C1P might generally have important functions in ERK-signaling in macrophages. Altogether, these results suggest that CERK deficiency attenuates MCP-1/CCR2 signaling in macrophages infiltrating adipose tissue, resulting in the suppression of inflammation in adipose, which otherwise leads to obesity and diabetes (Fig. 5).

4. Discussion

An increase in adiposity is suggested to cause a low grade chronic inflammation, leading to the activation of various immune

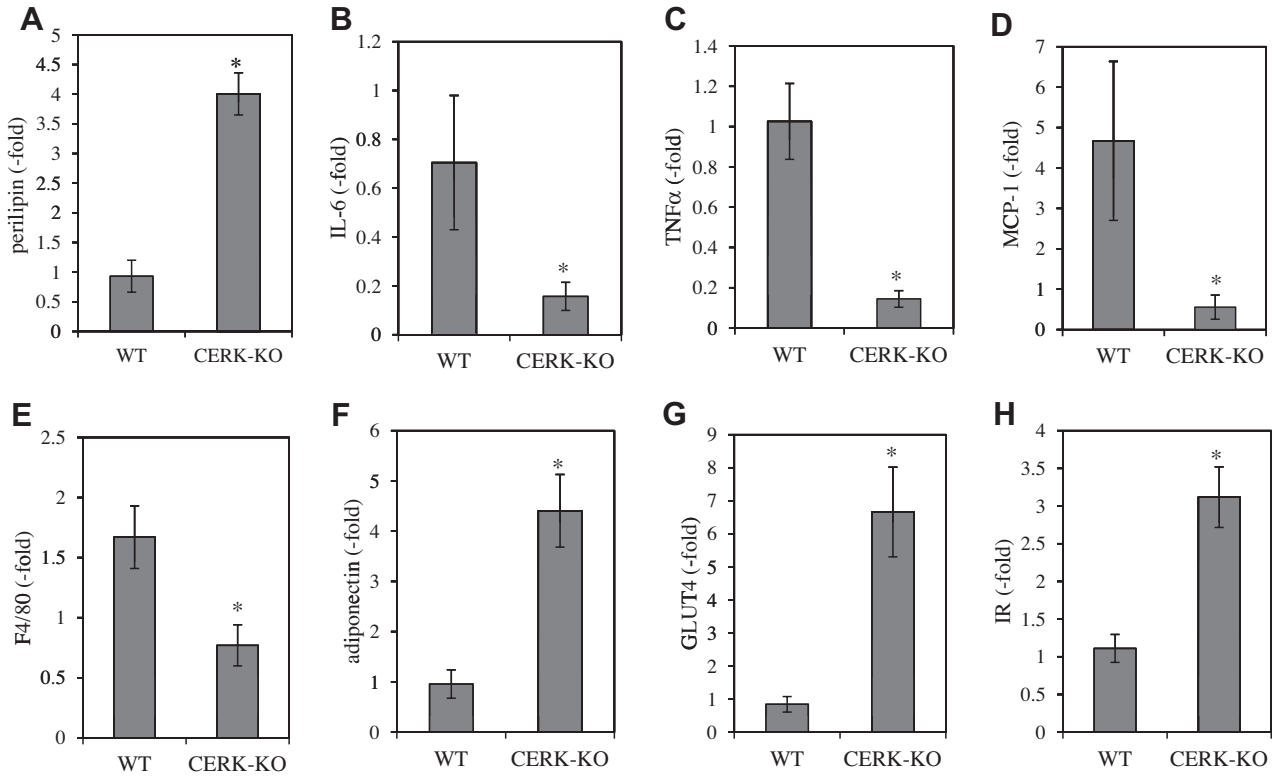

Fig. 2. Real-time PCR analysis of adipose tissue from WT and CERK-/- mice. Mice were fed a high fat diet for 15 weeks, then epididymal adipose tissue was harvested. RNA was isolated and real-time PCR for perilipin (A), IL-6 (B), TNFα (C), MCP-1 (D), F4/80 (E), adiponectin (F), GLUT4 (G), and IR (H) were performed as described under "Section 2". Data presented are the means ± S.D. for 5–11 mice. *, $P < 0.001$.

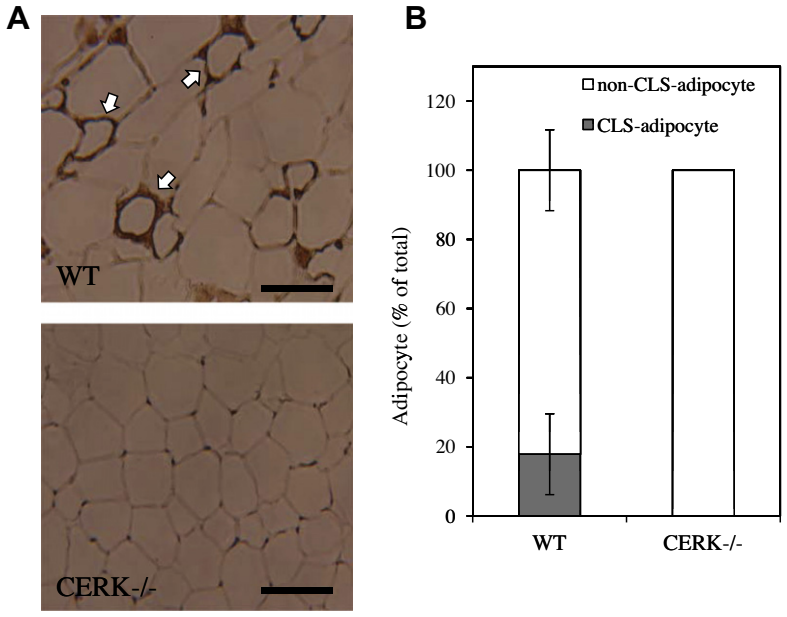

Fig. 3. Immunohistochemical analysis of crown-like structures in adipose tissue from WT and CERK-/- mice. (A) Sections of adipose tissues from WT and CERK-/- mice were stained for the macrophage marker F4/80 as described in Section 2. (B) Adipocytes having crown-like structures (CLR) were counted, and are presented as a percentage of the total adipocytes counted (a minimum of 1500 cells per tissue for WT ($n = 5$ mice) or CERK-/- ($n = 5$ mice) mice.

cells, and finally resulting in severe obesity and diabetes [4]. In this process, the infiltration of macrophages into adipose tissue is thought to be a crucial event, in which MCP-1 and its receptor CCR2 are key players [15]. Indeed, mice with targeted deletions in the genes for MCP-1 or its receptor CCR2 have dramatically decreased numbers of macrophages or CLS in adipose tissue, decreased inflammation in fat, and protection from high-fat diet-induced insulin resistance [19]. Thus, the inhibition of macrophage infiltration of adipose tissue is considered a potent therapeutic strategy for diabetes associated with obesity. In the study pre-

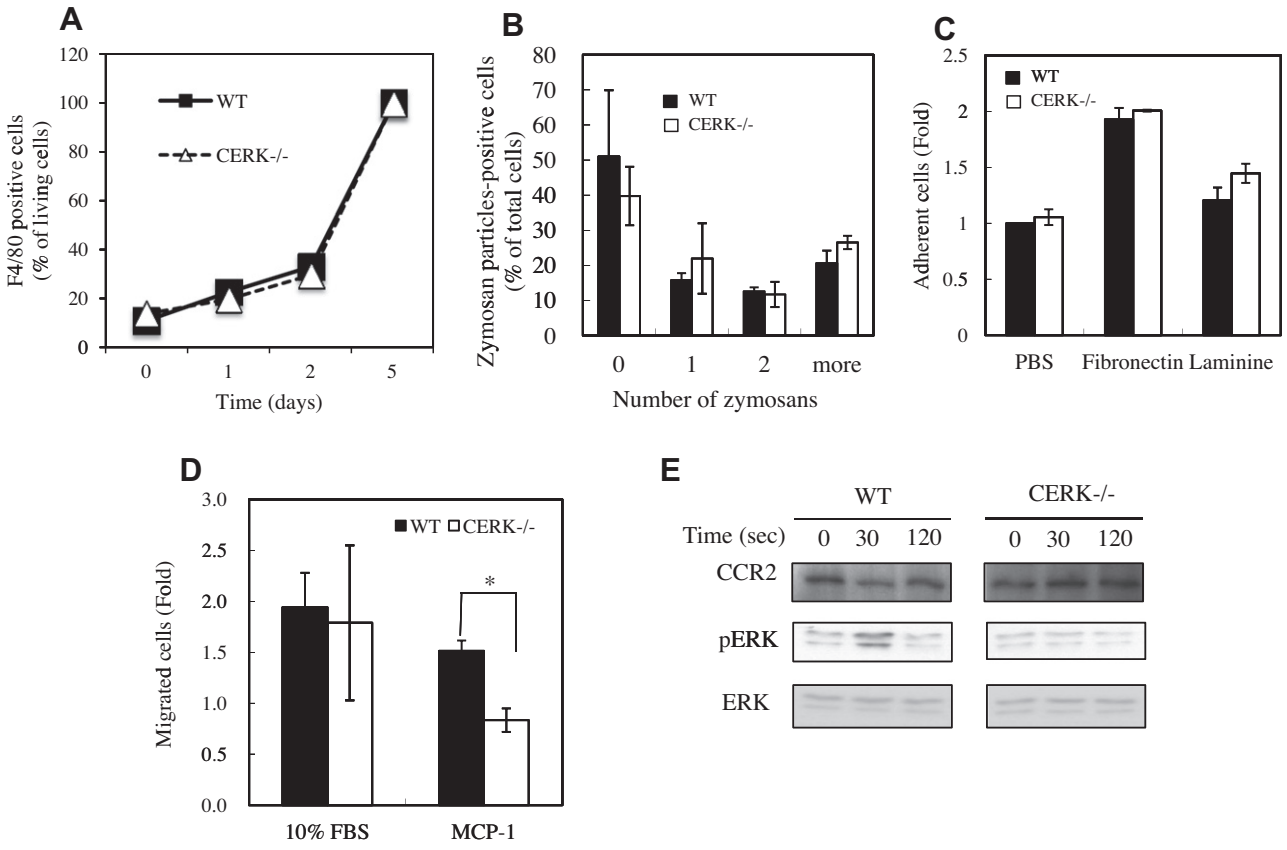

Fig. 4. Effect of CERK deficiency on macrophage cell functions. Bone marrow-derived macrophages (BMDM) were isolated from WT and CERK-/- mice as described in Section 2. (A) The differentiation levels of the BMDM were monitored by flow cytometry and staining for the macrophage marker F4/80. (B) Phagocytosis assays were performed using BMDM and counting the zymosans particles incorporated. (C) Adhesion to fibronectin and laminin of BMDM was examined. (D) Migration of BMDM toward serum or MCP-1 was examined using Boyden Chambers. The data are expressed as the fold increase over control experiments. (B–D) The data represent the means ± S.D. for 5–6 mice. *, $P < 0.01$. E, MCP-1/CCR2-mediated phosphorylation of ERK was examined using BMDM from WT or CERK-/- mice. After the BMDM were starved overnight, MCP-1 (final concentration 50 ng/ml) or FBS (final concentration 10%) was added to the medium, and the cells were lysed after 0, 30, or 120 s, and were subjected to Western blotting. Details of the experiments using BMDM are described in Section 2.

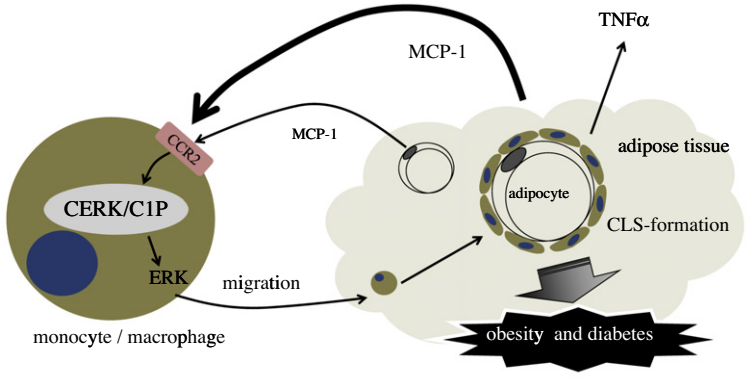

Fig. 5. Possible role of CERK in obesity and diabetes. CERK deficiency attenuates MCP-1/CCR2 signaling in macrophages infiltrating adipose tissue, and dramatically reduces numbers of macrophages or crown-like structures (CLS) in adipose tissue, resulting in the prevention of obesity and diabetes.

sented here, we demonstrated that CERK deficiency attenuates MCP-1/CCR2 signaling in macrophages infiltrating adipose tissue, resulting in the prevention of obesity and diabetes. Dr. Claro's group reported that ceramide kinase govern the biogenesis of lipid droplets [20], and we also found that CERK-/- mice have a greater number of small adipocytes than the WT mice do, indicating CERK is involved in the swelling process of adipocytes. Thus, not only the MCP1/CCR2-signaling in macrophages, but also the swelling process of adipocytes would be impaired in CERK-/- mice. Our findings might provide new insight into the development of pharmaceuticals against obesity and diabetes.

The number of published studies regarding sphingolipids and diabetes, and obesity is steadily increasing. For example, in obese ob/ob mice diminishing glycosphingolipid biosynthesis using AMP-DNM, an inhibitor of glucosylceramide synthase, reduced the number of adipose tissue macrophages and CLS and restored

insulin signaling [21]. A study in our lab revealed that sphingomyelin synthase 2 is involved in diet-induced obesity [7]. Now, sphingolipid metabolites, such as ceramide, C1P, and sphingosine, have been implicated in inflammatory responses in various sites of action [22]. Enzymes involved in ceramide-pathways might have potential as targets for drug discovery against diabetes.

Acknowledgements

This work was supported in part by a Grant-in Aid for Young Scientists (B) (23780344) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

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